REMARKS

Claims 1-27, 32, 33, and 36-38 were pending in the application, prior to the present amendment. The non-elected claims, 27, 32, 33, and 36-38, have been canceled. Figure 6 was objected to for including reference characters that are not mentioned in the specification. The specification was objected to for including trademarks that are not capitalized and sequences lacking sequence identification numbers. Claims 1, 12, 14, 15, and 19-23 were objected to due to informalities. Claims 1-26 were rejected for lack of sufficient written description and for lack of enablement, and claims 4 and 5 were rejected for indefiniteness. The objections and rejections are addressed as follows.

Drawings

Figure 6 was objected to for including reference characters that are not mentioned in the specification. This objection has been met by the present amendment to the description of Figure 6, on page 7 of the specification, to indicate what is meant by each of the so-called reference characters in the Figure. In particular, amino acids indicated by single letter code in the figure have been noted in the description in connection with the corresponding three-letter code (i.e., N210 is indicated as Asn210, D208 is indicated as Asp208, G240 is indicated as Gly240, and D242 is indicated as Asp242), and the notations of bond length in the figure (2.7, 2.8, and 3.3 Angstroms) have been indicated as such in the description. No new matter has been added.

Specification

The specification was objected to for including trademarks that are not capitalized. The specification has now been amended so that the trademarks are capitalized.

The specification was also objected to for including sequences at pages 39, 40, and 45 that lack sequence identifiers. These pages of the description have been amended to include sequence identifiers, accordingly. No new matter has been added.

Claim Objections

Claims 1, 12, and 19-23 were objected to on the basis that the letter "s" should be added to the word "repeat." The claims have been amended accordingly.

Claims 14 and 15 were objected to for including the abbreviations FIH and PHD, with the Examiner stating that the first use of any acronym should be followed by an explanation as to what is being abbreviated. Claim 14 has been amended to specify "factor inhibiting hypoxia-inducible factor" (FIH), and claim 15 has been amended to specify "prolyl hydroxylase domain" (PHD), accordingly. Support for these amendments can be found, for example, at page 2, lines 3-7. No new matter has been added.

Rejection of Claims 1-26 for Lack of Written Description

Claims 1-26 were rejected under 35 U.S.C. § 112, first paragraph as lacking adequate support in the written description. The Examiner suggested that removal of the phrase "all fragments thereof" from the claims would be sufficient to overcome the rejection (see page 6 of the Office Action). Claims including this phrase, 1, 12, and 19, have been amended to delete the phrase. Applicants thus request that this rejection be withdrawn.

Rejection of Claims 1-26 for Lack of Enablement

Claims 1-26 were rejected under 35 U.S.C. § 112, first paragraph for lack of enablement. The Examiner suggested that removal of the phrase "all fragments thereof" from the claims would be sufficient to overcome the rejection (see page 12 of the Office Action). As noted above, claims including this phrase, 1, 12, and 19, have been amended to delete the phrase. Applicants thus request that this rejection be withdrawn.

Rejection of Claims 4 and 5 for Indefiniteness

Claims 4 and 5 were rejected for indefiniteness, with the Examiner stating that there is no explanation in the specification as to the meaning of "2-5 A-d-R." In response, Applicants note that 2-5 A-d-R stands for 2-5 A(adenine)-dependent RNase, as explained in the specification at, for example, page 43, lines 20-21. Claim 4 has been amended accordingly. No new matter has been added.

Information Disclosure Statement

The Form PTO 1449 returned with the Office Action indicates that Bickel et al., Hepatol. 28:404-411, 1998, was not submitted and thus was not considered. In response, Applicants note that in PAIR the Bickel reference is the first reference listed as an "NPL document" on March 21, 2007. Another copy of the reference, as well as a further copy of the Form PTO 1449, is submitted herewith for the Examiner's convenience. Applicants respectfully request that the Bickel reference be considered and that the Examiner let the undersigned know if anything further is required on this matter.

CONCLUSION

Applicants submit that the claims are in condition for allowance, and such action is respectfully requested. If there are any charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: June 2, 2009

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Selective Inhibition of Hepatic Collagen Accumulation in Experimental Liver Fibrosis in Rats by a New Prolyl 4-Hydroxylase Inhibitor

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Fibrosis and cirrhosis of the liver are often the result of chronic liver damage by a variety of different agents. Pathological accumulation of collagen, disruption of the lobular structure, and impaired hepatocellular function frequently lead to systemic involvement and fatal complications. Drugs inhibiting collagen hydroxylation and accumulation are expected to improve this situation, making prolyl 4-hydroxylase (P4H), the key enzyme of intracellular collagen processing, a rational target for pharmacological intervention. S 4682, a novel inhibitor of purfied P4H (K_i = 155 nmol/L), reduced hydroxyproline (Hyp) synthesis in chicken embryo calvaria (IC_{50} = 8.2 μmol/L) and in cultured hepatic stellate cells (HSC) (IC_{50} = 39 μmol/L). S 4682 inhibited hepatic collagen hydroxylation *in vivo* after metabolic labeling with [^{14}C]proline. In the CCl_4 model of chronic hepatic injury, characterized by histologically and biochemically evident fibrosis and highly elevated levels of serum procollagen type III N-peptide, S 4682 reduced hepatic collagen accumulation, decreased prevalence of ascites, and lowered serum procollagen type III N-peptide (PIIINP) levels. The hepatic Hyp content of drug-treated animals was closely correlated with serum levels of PIIINP. S 4682 had no influence on Hyp content of heart, lung, and kidney. (HEPATOLOGY 1998;28:404-411.)

Chronic damage to the liver by a variety of causes frequently results in fibrogenesis, the increased deposition of extracellular matrix (ECM) proteins. The main component of the newly formed ECM is collagen.[1] Hepatic stellate cells (HSC), when transformed into myofibroblasts, are the major source of this newly synthesized collagen.[2,3] In the long run, the pathological accumulation of collagen disrupts the organ's lobular structure and impairs hepatic function, frequently accompanied by increased portal vein pressure and reduced hepatic blood flow. The end-stage of fibrogenesis is cirrhosis, a disorder for which no specific treatment is currently available. In spite of the complex regulatory events leading to fibrogenesis, the biosynthesis of collagen is a uniform process, making it a suitable target for therapeutic intervention.[2]

The crucial step of the intracellular collagen processing is the synthesis of hydroxyproline (Hyp) residues by the enzyme, prolyl 4-hydroxylase (EC 1.14.11.2; P4H).[4] Hyp residues stabilize the collagen triple helix. Underhydroxylated collagen is not stable at body temperature. It is retained and rapidly degraded inside the collagen-producing cells.

P4H has long been recognized as an ideal target for the pharmacological control of excessive collagen biosynthesis.[5] S 4682, a heterocyclic carbonyl-glycine derivative (Fig. 1), was designed as an analog of the enzyme's cosubstrate, 2-oxoglutarate. Its efficacy *in vitro* was shown using purified enzyme, intact cells, and organ cultures. The compound's inhibitory effect on P4H activity *in vivo* was studied by the determination of the Hyp content in collagen extracted from the liver after administration of [^{14}C]proline to rats. Antifibrotic activity of S 4682 could be demonstrated in a long-term experiment, inducing liver fibrosis in rats by chronic application of CCl_4.

MATERIALS AND METHODS

Purification and Assay of Enzyme Activity

P4H was purified from homogenized, 14-day-old chicken embryos by ammonium sulfate fractionation, 0% to 70% saturation, affinity chromatography on poly (L-proline) coupled to sepharose 4B, and diethyl aminoethyl (DEAE) chromatography as previously reported.[6,7] Determination of enzyme activity was performed as described previously.[8] (Pro-Pro-Gly)$_{10}$ × 9 H_2O, the synthetic substrate for P4H, was obtained from the Protein Research Foundation, Minoh, Osaka, Japan. 2-Oxo[5-^{14}C]glutarate was obtained from Amersham Buchler, Braunschweig, Germany. It was diluted to 100,000 dpm/0.005 μmol/L by mixing with unlabeled compound purchased from Merck, Darmstadt, Germany. Analysis of the kinetic data was performed using a commercially available computer program (Leatherbarrow RJ [1992], GraFit Version 3.0, Erithacus Software Ltd., Staines, UK).

Inhibition of P4H Activity Cell and Organ Culture

Chicken Embryo Calvaria. Hyp synthesis was assayed after metabolic labeling of chicken embryo calvaria with [U-^{14}C]-proline (NEN-Life Science Products, Bad Homburg, Germany) for 2.5 hours in the presence of various inhibitor concentrations. Determination

Abbreviations: ECM, extracellular matrix; HSC, hepatic stellate cells; Hyp, hydroxyproline; DEAE, diethyl aminoethyl; P4H, prolyl 4-hydroxylase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Pro, proline; ALT alanine transaminase; PIIINP aminoterminal propeptide of type III procollagen; AST aspartate transaminase; 2,4-PDCA pyridine-2,4-dicarboxylic acid; 2-PCA pyridine 2-carboxylic acid.

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of [^{14}C]-Hyp was performed by acidic extraction of collagen, amino acid analysis of the hydrolyzed protein, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously.[9,10] Under these conditions, the major part of the newly synthesized collagen is deposited in the ECM. Because of the short incubation time, an effect on cell proliferation is not to be expected. IC_{50} and K_i values were calculated using a commercially available computer program (Leatherbarrow RJ [1992], GraFit Version 3.0, Erithacus Software Ltd.).

HSC. Rat HSC were purified by *in situ* non-recirculating collagenase perfusion and low-speed gradient centrifugation using 25% Percoll (Pharmacia Biotech Europe GmbH, Freiburg, Germany).[11] Female Sprague-Dawley rats (Møllegaard Breeding and Research Centre A/S, Lille Skensved, Denmark) of 200 to 250 g body weight were routinely used as donor animals. HSC were cultured in Williams' E Medium containing 10% fetal calf serum, as well as gentamycin and amphotericin, in a humidified atmosphere containing 5% CO_2. HSC recovered from one liver were used to set up cultures of three 6-well plates (Corning Costar Germany, Bodenheim, Germany). For inhibition studies, cells were used 1 week after initiation of the cultures. At this stage, cell layers were confluent, and all cells were expressing α-smooth muscle actin and desmin as demonstrated by immunostaining (data not shown). Inhibition of cellular prolyl hydroxylation was assayed by using a dual-label method as previously published.[12] Briefly, newly synthesized collagen was labeled by incubating HSC in serum-free Eagle's minimum essential medium supplemented with 50 μg/mL ascorbic acid containing [4-^{3}H]-proline and [U-^{14}C-]-proline (NEN-Life Science Products) at the ratio and concentration as described.[12] Inhibitor was added at varying concentrations together with radioactivity, and the cells were incubated for 2.5 hours. Under these conditions, 80% to 90% of the newly synthesized collagen is detected in the cells and in the cell-associated matrix (data not shown). Because of the use of confluent cultures and short incubation time, an effect on cell proliferation is not to be expected. Then, cells were harvested and cellular protein was submitted to collagenase digestion to liberate collagenous peptides. Collagenase (Worthington Biochemical Corporation, Freehold, NJ) used in this assay was purified by column chromatography as described.[13] Inhibition of cellular prolyl hydroxylation was assessed by measuring the ratio of [4-^{3}H]-proline versus [^{14}C]-proline recovered in the collagenase digest. Calculation of IC_{50} values was performed using a commercially available computer program (Leatherbarrow RJ (1992), GraFit Version 3.0, Erithacus Software Ltd.).

Inhibition of P4H Activity In Vivo

Male and female Wistar rats weighing 150 to 180 g were injected intraperitoneally with 2 × 1 mL radiolabeled [U-^{14}C]-L-proline (2 × 50 μCi/mL, 200-300 mCi/mmol), purchased from Biotrend, Köln, Germany, in phosphate-buffered saline at time points 0 and 3 hours of the experiment. Seven rats, four males and three females, received S 4682 (3 × 100 mg/kg intraperitoneally, dissolved in saline in a volume of 5 mL/kg) at time points −0.15, 2, and 4 hours with respect to the application of radioactivity. Control animals, two males and one female, received the identical treatment with vehicle only (n = 3). Six hours after the first [^{14}C]-L-proline (time point 0) injection, the animals were killed, the livers were removed rapidly, shortly rinsed in saline, weighed, and frozen in liquid nitrogen. Purification of hepatic collagen followed standard procedures.[14,15] The frozen organs were thawed, homogenized, and centrifuged at 30,000 rpm for 30 minutes. All the following procedures were performed at 4°C. The pellet was digested five times for 6 hours with 10 mg pepsin per gram of liver tissue in 0.5 N acetic acid (pH 2.5). After the last digestion cycle, the solution was centrifuged at 40,000*g* for 30 minutes. The supernatants were adjusted to pH 8 and collected. The collagen fraction was precipitated with 30% ammonium sulfate (Merck, Darmstadt, Germany) at pH 8 for 24 hours and centrifuged (200,000*g* for 1 hour). The pellet was redissolved in 0.5 N acetic acid (pH 2.5) by stirring for 24 hours and subsequent centrifugation (100,000*g*, 1 hour). The resulting supernatant was again precipitated and centrifuged as described, washed in 70% ethanol, and redissolved in 1 mL 0.5 N acetic acid. The purity of the obtained collagen fraction was confirmed by SDS-PAGE and autofluorography. The material consisted of collagens type I, III, and V, as well as type IV collagen fragments, as determined by SDS-PAGE, Western blotting, and antibody staining. Contaminating noncollagenous proteins could not be demonstrated. The presence of laminin, a basement membrane protein containing a pepsin-resistant domain, was excluded by antibody staining using an anti-rat yolk sac laminin antibody (data not shown), demonstrating the selectivity of the precipitation steps.

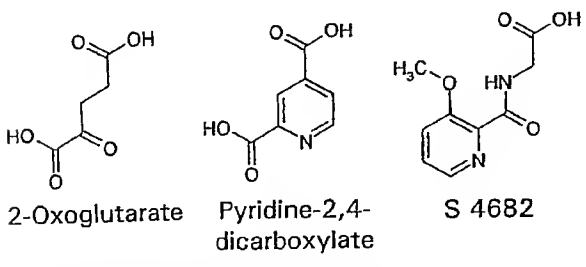

FIG. 1. Chemical structure of 2-oxoglutarate, 2,4-PDCA, and S 4682, illustrating the chemical similarities of the compounds.

Sample Preparation for Amino Acid Analysis

Vacuum-dried samples of purified hepatic collagen were hydrolyzed in gas phase (6 N hydrochloric acid) at 150°C for 15 minutes employing a microwave apparatus (Type MDS-2000, CEM, Kamp-Lintfort, Germany). The hydrolyzed samples were dissolved in sample buffer (Eppendorf, Hamburg, Germany) and stored at −80°C until use. Amino acid analysis was performed on a LC 3000 amino acid analyzer (Eppendorf-Biotronik, Hamburg, Germany). Calibration of the analyzer was performed using Benson Type H standard (Eppendorf). Separations were performed employing a modified protocol for serum amino acids as provided by Eppendorf; norleucine was used as an internal standard. Proline (Pro) and Hyp peaks were collected with a sample collector and counted in a scintillation counter. Activity of P4H is expressed by the ratio of radioactive Hyp/Pro, and calculated by the formula: Hyp/(Hyp + Pro).

Experimental Liver Fibrosis

Male Wistar rats with an initial body weight of 212 to 320 g (Breeding plant: Research Institute for Pharmacy and Biochemistry, Konárovice, Czech Republic) were used. Experimental liver fibrosis was produced as described elsewhere.[16,17] Briefly, all animals, except the controls, received CCl_4, 1 mL/kg by gavage, dissolved in olive oil 1:1 (vol/vol) twice weekly, for a period of 9 weeks. S 4682 was given concomitantly with CCl_4 twice daily at a dose of 60 mg/kg intraperitoneally, dissolved in saline and administered in a volume of 2 mL/kg. Rats treated with CCl_4 only received saline. The animals were kept under standard conditions as described below. The day/night rhythm was lights on from 6:30 AM to 6:30 PM, room temperature was 22 ± 2°C, and the relative humidity was 60% ± 10%. The rats were fed a standard rat chow ST-1 from Velaz, Lysá and Labem, Czech Republic, and water *ad libitum*. After 9 weeks, the end of the experiment (the last CCl_4 application was given 5 days before killing the animals), the animals were anesthetized with ether and exsanguinated through the caval vein. The liver was removed and weighed. A sample from the left lobe of the liver of approximately 0.5 g was frozen for determination of Hyp content of the liver. The tip of the heart, the lower lobe of the right lung, and the entire left kidney were frozen for determination of Hyp content of the organs.

Collagen content of liver, heart, lung, and kidney was measured as Hyp content of the organs by high-performance liquid chromatography as described.[18] Hyp content was expressed in nanograms per milligram of wet weight or as the Hyp/Pro ratio, the latter being independent from the organ's weight. Bilirubin, alanine transaminase (ALT), alkaline phosphatase, albumin (commercially available kits from Behring, Marburg, Germany, and Boehringer, Mannheim, Germany), total bile acids,[19] and the aminoterminal propeptide of type III procollagen (PIIINP) were determined in the serum.[20]

Histology

Histological analysis of the sirius red-stained liver sections was performed in a blinded fashion; the severity of fibrosis was scored with six degrees of freedom as defined below: 0 = normal liver histology; 1 = tiny and short bundles of collagen growing from portal and/or central areas into the lobules; in most parts of the liver, the collagen content seems to be normal; 2 = more than only very few bundles start to touch and to connect central and portal areas; in rare cases, single hepatocytes next to the portal areas are separated by collagen; separation by growing to pseudolobules has not really started; 3 = pseudolobular transformation starts in more than one or two areas; septa grow broader and some of the nodules get a rounded shape caused by proliferation of hepatocytes; 4 = nodular transformation and rounded shape can be seen in the whole slice; 5 = excessive formation and deposition of connective tissue with subdivision of the regenerating nodules, isolation of small groups of hepatocytes, and development of scars.

Subchronic CCl_4-Induced Liver Injury

Male Wistar rats (Hoe:WISKf (SPF 71, Wistar) (breeding plant: Hoechst AG, Hattersheim, Germany), with an initial body weight of 120 ± 13 g, were randomly divided into five treatment groups as shown in Fig. 2.

S 4286 and malotilate were dissolved in saline and administered once daily in a volume of 2 mL/kg intraperitoneally. CCl_4, 1 mL/ kg per os, dissolved in olive oil 1:1 (vol/vol), was given at days 1 and 2 as shown in Fig. 2. The animals were kept under standard conditions (day/night rhythm from 6 AM to 6 PM, 22°C room temperature; standard pellets of Altromin 1321 [Altromin GmbH, Lage/Lippe, Germany]) and water *ad libitum*. Blood (100 µL) was withdrawn in slight ether anesthesia from the retro-orbital plexus at days 0, 2, 3, and 4. At day 6, the end of the experiment, the animals were anesthetized with ether and exsanguinated through the caval vein. As a parameter of hepatic injury, aspartate transaminase (AST) and ALT (commercially available kits from Boehringer, Mannheim, Germany) were measured in serum withdrawn at the days indicated above.

Animal Care

Ethical approval for the animal experiments had been obtained from the Department of Veterinary Affairs of the Regierungspräsidium Darmstadt, Germany (14.06.1993). All animals received humane care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals*, prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23, revised 1985).

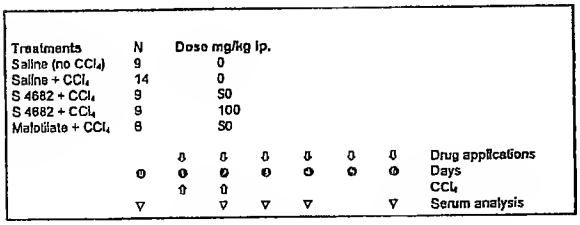

FIG. 2. Experimental design of subchronic liver injury.

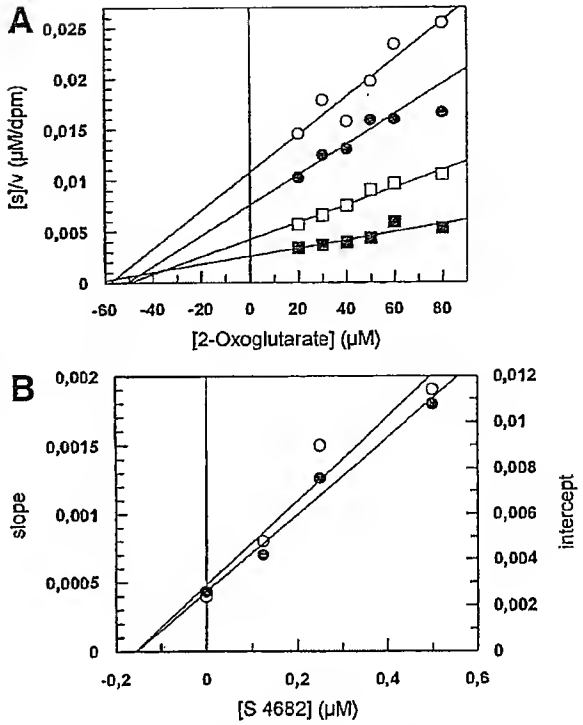

FIG. 3. (A) [s]/v versus [s] plot for the inhibition of P4H by S 4682 using 2-oxoglutarate as the variable substrate. [s], concentration of substrate; v, reaction velocity. The plot is best described as noncompetitive. The concentrations of S 4682 were: (○) = 0.5 µmol/L; (⊖) = 0.25 µmol/L; (□) = 0.125 µmol/L; (▣) = uninhibited samples. Each point is the average of three independent experiments. (B) Secondary transformation of the data presented in (A). The K_{is} value is calculated from the slopes of the regression line of (A) (○). The K_{ii} value is calculated from the intercepts of the regression line of (A) (●).

Statistics

Testing for significant differences was basically performed by nonparametric procedures. Quantal treatment responses were tested using the χ^2 test.[21] Correlations between parameters were tested in the case of normal distribution by simple analysis of linear regression, or otherwise with the nonparametric rank correlation tests according to Spearman[22] or Mann-Whitney. Testing for normal distribution was performed using a commercially available software

TABLE 1. Inhibitory Effects of 2-Oxoglutarate Analogs on the Purified Enzyme (P4H) and in Cell and Organ Culture

Compound	Purified P4H IC_{50} (µmol/L)	Chicken Calvaria IC_{50} (µmol/L)	Rat HSC IC_{50} (µmol/L)
2-PCA	145 ± 23	ND	ND
(n)	(6)		
2,4-PDCA	10.4 ± 2.96	750	>1,000
(n)	(10)	(1)	(3)
S 4682	0.284 ± 0.09	8.2 ± 1.2	39 ± 5
(n)	(3)	(4)	(3)

NOTE. Results are means ± SD.
Abbreviations: n, number of experiments; ND, not determined.

TABLE 2. Effects of S 4682 on Mortality, Body and Liver Weight, and Liver-Related Serum Parameters After 9 Weeks of Application of CCl_4

Treatment	Dose* (mg/kg)	N_c/N_e (mortality)	Body Weight (g)	Liver Weight (g)	Bilirubin (μmol/L)	Bile Acids (μmol/L)	ALT (U/L)	Alkaline Phosphatase (U/L)
Control	0	10/10 (0%)	425 ± 66.9	13.9 ± 2.61	2.00 ± 0.50	8.48 ± 8.40	27.5 ± 10.9	156 ± 57.5
CCl_4	0	80/80 (0%)	370 ± 43.3†	14.3 ± 3.12	4.34 ± 3.93†	81.3 ± 87.9†	83.1 ± 51.7†	269 ± 117†
CCl_4 + S 4682	60	60/58 (3.4%)	373 ± 38.9†	14.8 ± 2.32	2.83 ± 2.21‡	40.8 ± 51.4†§	59.0 ± 29.5†§	195 ± 72.7†‡

NOTE. Results are means ± SD.
Abbreviations: N_e, number of animals entering the study; N_c, number of animals completing the study.
*Twice daily intraperitoneally.
†$P < .05$ vs. control.
‡$P < .05$ (CCl_4 + S 4682 vs. CCl_4).
§$P < .01$ (CCl_4 + S 4682 vs. CCl_4).

(Sigma Stat [1995], Version 1.0, Jandel Scientific Corporation, Erkrath, Germany).

RESULTS

Isolated Enzyme. S 4682 differed from other structural analogs of 2-oxoglutarate,[50] such as pyridine-2,4-dicarboxylic acid (2,4-PDCA) and pyridine 2-carboxylic acid (2-PCA) by inhibiting P4H in a noncompetitive fashion with respect to the cosubstrate, 2-oxoglutarate (Fig. 3A). Secondary transformation (Fig. 3B) revealed a K_{ii} value (calculated from the intercepts of Fig. 3A) and a K_{is} value (calculated from the slopes of Fig. 2A) of 157 nmol/L and 153 nmol/L, respectively. The corresponding IC_{50} values were 0.250 μmol/L for S 4682, 10.4 μmol/L for 2,4-PDCA, and 145 μmol/L for 2-PCA (Table 1).

Inhibition of P4H in Cell and Organ Cultures. S 4682 inhibited Hyp synthesis in chicken embryo calvaria with an IC_{50} value of 8.2 μmol/L. Total incorporation of radioactivity was not reduced up to concentrations of 200 μmol/L. Analysis of procollagen processing by SDS-PAGE showed the characteristic inhibition of procollagen conversion demonstrated previously.[9,10] 2,4-PDCA inhibited Hyp synthesis in chicken embryo calvaria dose-dependently with an IC_{50} = 750 μmol/L.

In rat HSC in culture, [^{14}C]-Hyp synthesis was inhibited in a dose-dependent fashion by S 4682 with an IC_{50} value of 39 μmol/L. 2,4-PDCA was much less potent with an IC_{50} value > 1,000 μmol/L (Table 1).

Inhibition of P4H In Vivo. P4H activity was measured indirectly as the amount of [^{14}C]-L-Hyp generated from [^{14}C]-L-proline incorporated into hepatic collagen of healthy rats within 6 hours. Under these acute experimental conditions, administration of 3 × 100 mg/kg S 4682 intraperitoneally decreased the formation of [^{14}C]-Hyp in hepatic collagen. The ratio Hyp/(Hyp + Pro) dropped significantly from 0.47 ± 0.013 to 0.42 ± 0.026 (means ± SD), giving evidence that the drug can decrease the enzyme's activity in the liver of intact healthy animals *in vivo*.

Experimental Liver Fibrosis. Liver fibrosis in rats was generated by long-term treatment with CCl_4. After 9 weeks of CCl_4 treatment, hepatic function was considerably impaired. Bilirubin, total bile acids, ALT, and alkaline phosphatase in serum increased by 117%, 856%, 201%, and 72%, respectively. Serum albumin (−12%) and body weight (−13%) decreased compared with normal untreated male, age-matched, control animals (Tables 2 and 3). Fifteen of 80 rats presented visible ascites, all but 1 having serum albumin levels below 25 g/L. PIIINP in serum rose by 352%, and Hyp content of the liver increased by 325% (Table 3). Distribution analysis of Hyp values with increasing classes of 200 ng/mg each in CCl_4-treated animals revealed a non-normal distribution (data not shown). A close correlation of PIIINP in serum versus Hyp content of the liver could be demonstrated in the animals having received only CCl_4 ($r = .712$; $P < .001$; $n = 80$). S 4682, given twice daily (60 mg/kg concomitantly with CCl_4), significantly decreased bilirubin (−64%), total bile acids (−65%), ALT (−43%), and alkaline phosphatase (−65%) in serum. No significant difference in mortality was observed between CCl_4 and CCl_4 + S 4682 (Table 2). PIIINP levels in serum were reduced by 35%, and Hyp content of the liver dropped significantly by 29% (Table 3). This decrease was mainly caused by the less-severe degrees of liver fibrosis of drug-treated animals (Figs. 4 and 5).

The number of animals with ascites was significantly lower (−78%) in the drug-treated animals, i.e., 2 of 58 in the drug-treated group, compared with 15 of 80 in the CCl_4-treated group (Table 3). Again, a very close correlation between PIIINP levels in serum and Hyp content of the liver could be demonstrated ($r = .706$; $P < .001$; $n = 58$) in the drug-treated rats. Taking a low cutoff in PIIINP at 8.1 ng/mL (mean + 3 SD of the untreated control group) (Table 3), 12% of the animals on drug were below that level, but only 1.3% in the CCl_4 group. Taking a high cutoff in PIIINP > 40 ng/mL, only 1.7% of the rats on drug, but 11.3% of the rats in the

TABLE 3. Effects of S 4682 on Liver Hyp, Serum PIIINP, Serum Albumin, and Ascites After 9 Weeks CCl_4 Application

Treatment	Dose* (mg/kg)	N_c	Hyp (ng/mg)	Total Hyp (mg)	PIIINP (ng/mL)	Albumin (g/L)	Ascites (%)
Control	0	10	177 ± 30.3	2.48 ± 0.7	5.66 ± 0.80	27.8 ± 5.16	0
CCl_4	0	80	753 ± 324†	10.35 ± 4.11†	25.8 ± 15.3†	24.5 ± 4.42	18.8
CCl_4 + S 4682	60	58	588 ± 259†§	8.47 ± 3.42†§	18.7 ± 10.7†§	24.2 ± 2.80†	3.4§

NOTE. Results are means ± SD.
Abbreviations: N_c, number of animals completing the study.
†$P < .05$ vs. control.
‡$P < .05$ (CCl_4 + S 4682 vs. CCl_4).
§$P < .01$ (CCl_4 + S 4682 vs. CCl_4).

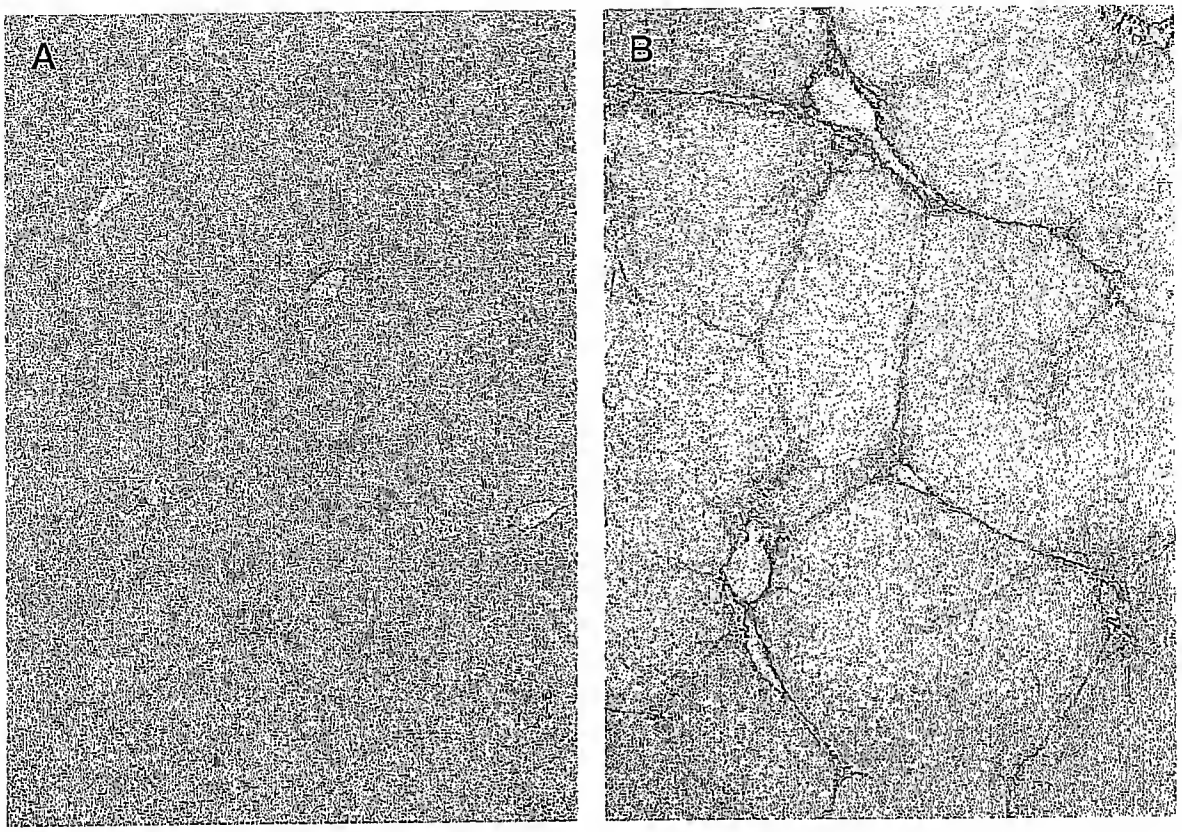

FIG. 4. Histological appearance of normal liver (A), the liver after 9 weeks of application of CCl_4 (B) (score 3-4), and after CCl_4 with concomitant treatment with S 4682 (C) (score 2-3). (Sirius red; original magnification ×100.)

CCl_4 group, had PIIINP values above 40 ng/mL, the difference being significant ($P < .02$). The findings of this analysis are in accordance with the results of the histological observations. Long-term treatment (9 weeks) with CCl_4 alone had no significant effects on Hyp content of the heart, lung, or kidney, or the corresponding Hyp/Pro ratios. S 4682 did not influence Hyp content of the heart, lung, or kidney (Table 4).

Subchronic Injury to the Liver. Injury of the liver in male Wistar rats was achieved by administration of CCl_4 on 2 consecutive days (days 1 and 2 of the experiment). This treatment caused a maximal 12- and 33-fold increase of serum ALT and AST, respectively, 1 day after the final CCl_4 administration (day 3 of the experiment). From these peak levels, serum ALT and AST decreased continuously. Concomitant application of S 4682 from day 1 to day 6 (50 and 100 mg/kg intraperitoneally daily) had no significant effect on the CCl_4-induced raise in transaminases in serum. However, malotilate (50 mg/kg intraperitoneally daily), given also from day 1 to day 6, significantly decreased this CCl_4-induced elevation of AST and ALT in serum by 53% and 57% at day 3, and by 33% and 30% at day 4, respectively (Table 5). At day 6, the end of the experiment, transaminases in serum returned to normal values in all groups.

DISCUSSION

Excessive deposition of collagen in the liver is a frequent result of chronic injury to the liver by a number of causative agents, with the irreversible end-point of cirrhosis. Cirrhosis, with its known serious and frequently lethal complications, is a major cause of death, ranking at number 5 to 6 of all deaths in the industrialized countries of Europe, North America, and Japan.

The complex mechanisms and events leading to deposition of collagen in the ECM provide numerous possibilities for therapeutic intervention. We have focused our research on the direct interference with collagen biosynthesis via inhibition of the enzyme, P4H (see above). The most advanced and successful approach so far has been using small molecules acting as inhibitors of the enzyme's cosubstrate, 2-oxoglutarate.[5]

The novel P4H inhibitor, S 4682, directly inhibited purified P4H, being substantially more potent than the structurally related 2,4-PDCA and 2-PCA at the enzyme level. Based on the IC_{50} values, S 4682 was 42 times more potent than 2,4-PDCA and 580 times more potent than 2-PCA (Table 1).

In chicken calvaria culture, S 4682 inhibited P4H with an IC_{50} value of 8.2 μmol/L, proving that the compound is able

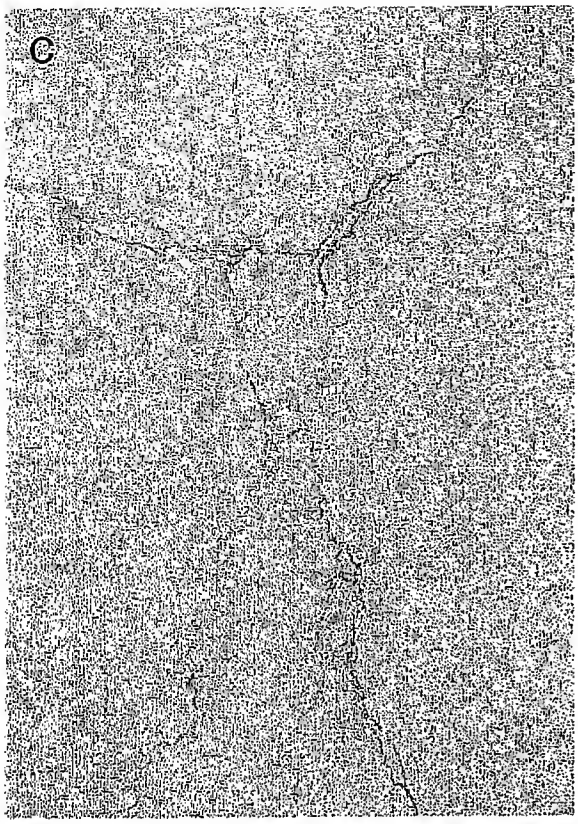

FIG. 4. (Cont'd.)

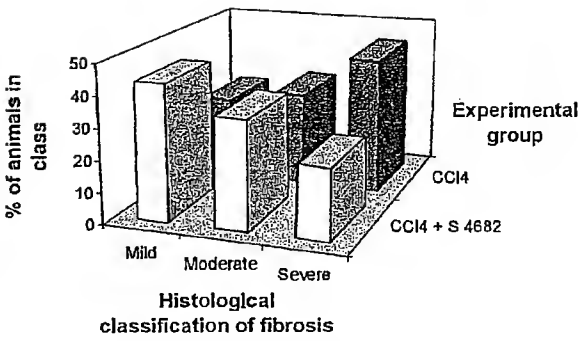

FIG. 5. Effect of S 4682 on liver fibrosis in CCl_4 fibrotic rats based on histological examination. *Dark columns*, rats treated with rats CCl_4 only (n = 80); *light columns*, rats treated with CCl_4 + S 4682 (n = 58). The ordinate shows the percentage of rats with histologically mild (score 1 and 2), moderate (score 3), and severe fibrosis (score 4 and 5). The percentage of rats with individual histological scores from 1 to 5 was 3.8, 22.5, 30, 27.5, and 16.3 in the CCl_4 group (mean ± SD, 3.3 ± 1.107), and 10.3, 32.8, 34.5, 20.7, and 1.7 in the CCl_4 + S 4682 group (mean ± SD, 2.71 ± 0.973), respectively. The difference between the groups was statistically significant ($P = .022$). There were no rats with histologically normal liver in either the CCl_4 or the CCl_4 + S 4682 groups.

to achieve access to P4H by penetrating the cellular membranes more effectively than 2,4-PDCA. S 4682 also decreased P4H activity in cultured rat HSC, demonstrating that the compound is able to enter the cells that produce collagen under fibrogenetic stimulus. The IC_{50} value for S 4682 was 39 μmol/L, being at least 26 times more potent than 2,4-PDCA (Table 1).

In an acute (6-hour) experiment in rats, formation of Hyp in newly synthesized liver collagen was significantly reduced, in accordance with the concept that S 4682 acts as an inhibitor of P4H *in vivo*. In a long-term experiment (9 weeks) producing liver fibrosis in rats by chronic application of CCl_4, S 4682 reduced collagen accumulation in the liver and PIIINP in serum significantly, and attenuated the morbidity of the animals as documented by the striking reduction of the occurrence of ascites (Table 3). We assume that the beneficial effects on the variables of liver function such as bilirubin, bile acids, and alkaline phosphatase in serum (Table 2) are secondary effects, resulting from the reduction of the fibrogenetic process.

To ensure that the decrease of serum ALT (Table 2) with S 4682 does not simply reflect an interference of the compound with the toxic effects of CCl_4, we investigated this possibility in a subchronic experiment, measuring the CCl_4-induced elevation of ALT and AST in serum as indicators of parenchymal liver cell injury. Transaminases in serum increased by an average of 2,250%. Concomitantly administered S 4682 had no effect on this elevation, whereas malotilate significantly decreased transaminases (Table 5). Our findings with malotilate are in agreement with the results of other investigators after short- and long-term treatment with CCl_4,[23-26] interpreting the effects of malotilate on collagen biosynthesis in CCl_4-induced liver fibrosis as caused by the reduction of postnecrotic inflammation.[26]

It has been shown[27,28] that serum collagen fragments, especially PIIINP, are predictive markers in different models of experimental liver fibrosis in rodents. The present data

TABLE 4. Hyp Content of Different Organs After 9 Weeks of Application of CCl_4

	Heart		Lung		Kidney	
Treatment (n)	Hyp*	Hyp/Pro†	Hyp*	Hyp/Pro†	Hyp*	Hyp/Pro†
Control (10)	616 ± 36	90.1 ± 6.91	3.76 ± 1.67	358 ± 20.3	620 ± 69	106 ± 8.61
CCl_4 (80)	627 ± 47	94.1 ± 8.64	3.19 ± 0.643	346 ± 20.2	609 ± 94	103 ± 9.09
S 4682‡ (58)	614 ± 88	92.1 ± 11.7	3.11 ± 0.325	342 ± 18.8	661 ± 335	112 ± 55.2

NOTE. Results are means ± SD.

*Hyp content of the organs (ng/mg wet tissue weight).

†Hyp/Pro ratio × 1,000.

‡60 mg/kg intraperitoneally twice daily.

TABLE 5. Aminotransferases in Serum Following Subchronic Liver Injury With CCl_4

	Control	CCl_4	CCl_4 + S 4682	CCl_4 + S 4682	CCl_4 + Malotilate
N	9	14	9	9	6
Dose mg/kg	0	0	50	100	50
AST (U/L)					
Day 0	52 ± 6.2	48 ± 8.3	58 ± 12	64 ± 8.4	67 ± 13
Day 2	48 ± 7.4	385 ± 227†	303 ± 87†	338 ± 82†	452 ± 144†
Day 3	52 ± 6.2	1,633 ± 1,072†	1,384 ± 1,113†	1,644 ± 936†	764 ± 690†‡
Day 4	51 ± 13	186 ± 94†	182 ± 61†	214 ± 77†	125 ± 139†
Day 6	62 ± 5.2	64 ± 8.3	67 ± 15	52 ± 6.0	67 ± 7.6
ALT (U/L)					
Day 0	48 ± 3.6	34 ± 4.2	40 ± 5.5	47 ± 7.6	41 ± 8.8
Day 2	43 ± 7.0	144 ± 89†	112 ± 37†	152 ± 46†	125 ± 39†
Day 3	44 ± 6.6	443 ± 421†	315 ± 207†	456 ± 273†	191 ± 149†‡
Day 4	46 ± 5.2	98 ± 58†	86 ± 31†	112 ± 41†	69 ± 17†
Day 6	47 ± 5.0	44 ± 9.0	45 ± 8.5†	42 ± 4.6	42 ± 11

*Dose given intraperitoneally once daily.
†$P < .05$ vs. time-corresponding control.
‡$P < .05$ malotilate vs. CCl_4.

show for the first time that PIIINP was closely correlated ($r = .706$; $P > .001$) to Hyp in the liver under the influence of a P4H inhibitor, suggesting that the efficacy of such compounds can be monitored by a surrogate variable like PIIINP in serum. A large number of articles, reviewed in Schuppan,[29] have shown that PIIINP in patients is closely related to the degree of liver fibrosis as measured by clinical variables or histological analysis of liver biopsies. The first report on the decrease of elevated serum PIIINP levels by drug treatment in a clinical situation came from a study with patients suffering from chronic hepatitis C. Eight patients were treated with interferon alfa for 1 year. Six of them had a sustained clinical response to treatment with concomitant normalization of serum PIIINP.[30]

In an earlier study using the toxic P4H inhibitor, vineomycin A1, in pig serum-induced liver fibrosis in rats, the antifibrotic effects (measured as Hyp of the liver) correlated significantly ($r = .830$) with mortality in the treatment groups,[31] suggesting that the decrease in Hyp in this study is caused by the death of the most severely ill animals. The compound also inhibited collagen synthesis in tissues other than the liver.[32,33] In a clinical situation, inhibition of collagen synthesis must be restricted to the affected organ to avoid serious side-effects. By contrast, S 4682 did not influence collagen content of heart, lung, and kidney (Table 4) at a dose of S 4682 that produced highly significant reduction of collagen accumulation in the liver. It is the first compound whose antifibrotic properties in experimental liver fibrosis induced by long-term treatment with CCl_4 can be attributed exclusively to inhibition of P4H activity. These findings support the concept that direct inhibition of P4H is a promising approach toward reducing pathological accumulation of collagen in the liver.

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